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A pH induced two-dimensional crystal of membrane-bound Na^+,K^+-ATPase of dog kidney

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Two-dimensional crystals of membrane-bound Na^+,K^+-ATPase were formed in acidic media and their qualities were investigated by electron cryo-microscopy as well as by conventional electron microscopy. At pH 4.8 in sodium citrate buffer, the best crystallization condition, more than 80% of membranes formed crystals. The high ratio allowed high-resolution images to be taken by electron cryo-microscopy. Image processing revealed that they had unique lattice constants (a = 108.7 Å, b = 66.2 Å, γ = 104.2°) and had few defects in the crystalline arrays. The reconstituted Fourier map of the ice-embedded crystal showed that there are two high contrast parts in one unit cell.

Sodium potassium adenosine triphosphatase (Na^+,K^+-ATPase); Two-dimensional crystallization; Ice-embedding technique; Electron cryo-microscopy; Computer assisted image processing

1. INTRODUCTION

Na^+,K^+-ATPase (sodium potassium adenosine triphosphatase) is a membrane protein of animal cells, which exchanges three interior sodium ions and two exterior potassium ions across the cell membrane during the coupled hydrolysis of one ATP molecule (for reviews, see [1] and [2], etc.). The functional unit of enzymatic activity is $\alpha\beta$-protomer, composed of α- and β-subunits, with a molecular weight of about 150 kDa [3].

Purified membrane-bound Na^+,K^+-ATPase crystallizes in a two-dimensional (2D) array in the presence of sodium monovanadate/ammonium monovanadate and magnesium [4], inorganic phosphate, magnesium and manganese [5], phospholipase A_2 [6], or cobalt-tetrammine-ATP [7]. However, the 2D crystals formed with these reagents are not suitable for high resolution structure analysis due to the following three problems: first, only a small number of the membranes crystallize; second, there are too many crystalline defects; and finally, the lattice constants differ among the crystals. These problems impeded the application of electron cryo-microscopy to the 2D crystals of Na^+,K^+-ATPase; cryo-techniques are indispensable to high resolution structure analysis using electron microscopy [8,9], and requires a low electron dose in the search for a good crystal because of its sensitivity to irradiation damage, that results in requirement of random shooting for imaging. Consequently, a high ratio of good crystals becomes essential in the case of electron cryo-microscopy of frozen hydrated specimens.

Here we report a novel method for the 2D crystallization of membrane-bound Na^+,K^+-ATPase in acidic media containing either sodium citrate, sodium acetate, or sodium phosphate. This method solves all the aforementioned problems. More than 80% of the membranes are crystallized; all the crystals had unique lattice conditions (a = 108.7 Å, b = 66.2 Å, γ = 104.2° and a symmetry of p2 two-sided plane group) and few crystalline defects.

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2. MATERIALS AND METHODS

Membrane-bound Na^+,K^+-ATPase was prepared from the outer medulla of dog kidney by the method of Jørgensen [10], with modifications described by Hayashi et al. [3]. The specific ATPase activity measured as mentioned later was 47.6 U/mg protein (1 U is defined as μmol P_i/min) at 37°C.

To crystallize the protein, the membrane-bound Na^+,K^+-ATPase was suspended in 20 mM imidazole and 16 mM HEPES, pH 7.6 at 0°C. Then, the suspension was dialyzed against 50 mM sodium citrate, 150 mM sodium acetate, or 150 mM sodium phosphate, at pH values between 3.0 and 6.0, on ice, using glass tubes, as described in Kühlbrandt [11]. As the medium inside the tube was completely exchanged with each medium outside in a few days, the specimens were transferred from the glass tubes to microtubes and reserved on ice. After negative staining with 1% uranyl acetate, they were examined with a JEOL 100CX electron microscope operated at 100 kV. Images of the crystals were recorded at a magnification of 40,000. The best specimen was obtained in 50 mM sodium citrate at pH 4.8, and the images of this specimen were also recorded by a JEOL 4000SFX (electron cryo-microscope) [12] after being embedded in ice. The mi-

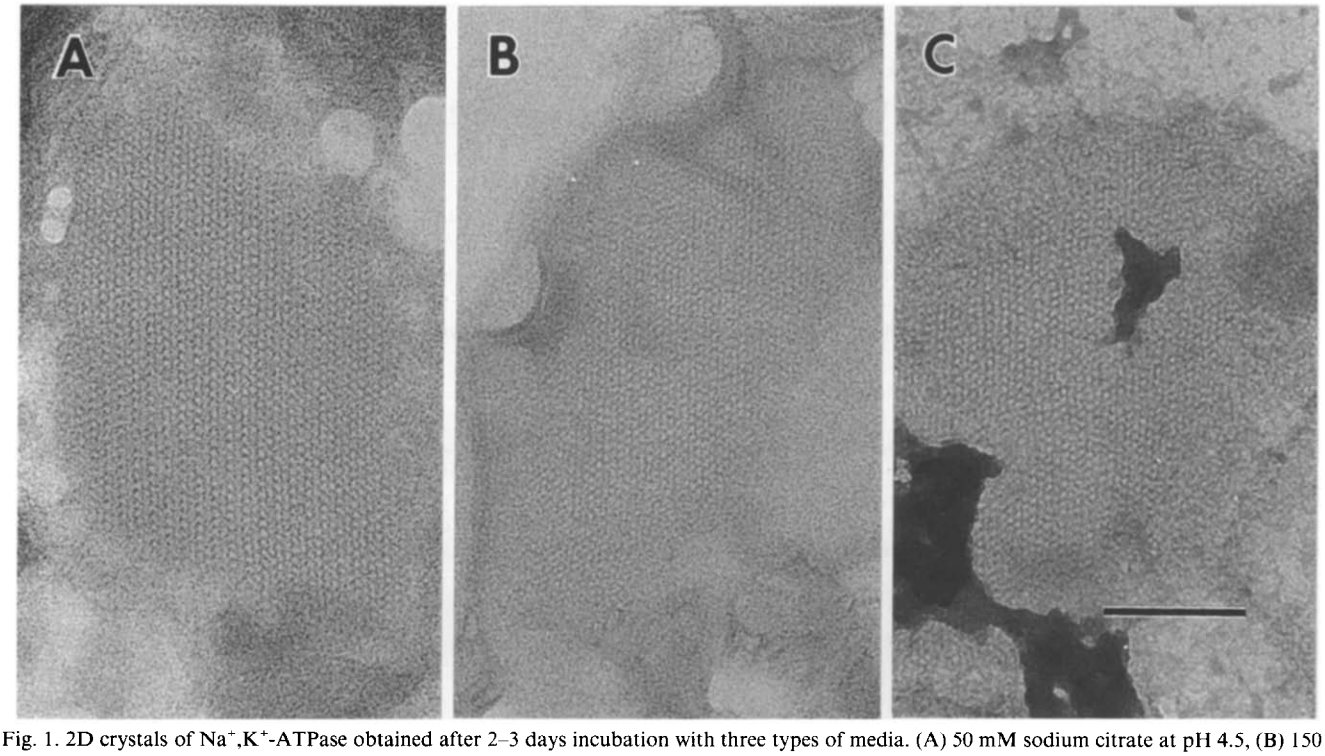

Fig. 1. 2D crystals of Na^+,K^+-ATPase obtained after 2–3 days incubation with three types of media. (A) 50 mM sodium citrate at pH 4.5, (B) 150 mM sodium acetate at pH 4.8, and (C) 150 mM sodium phosphate at pH 4.6. Bar = 0.1 μm.

croscopy was performed at a stage temperature of 4 K, an acceleration voltage of 400 kV, and the same magnification. Images of the negatively stained and the ice-embedded 2D crystals were digitized with a Perkin-Elmer micro-densitometer 1010GM, and were processed on a VAX station 4000 using computer programs essentially developed by Henderson et al. [8] and transferred from EMBL by courtesy of Dr. Kühlbrandt

The ATPase activity of the Na^+,K^+-ATPase was measured as follows after incubation in either 20 mM imidazole and 16 mM HEPES at pH 7.6, or 50 mM sodium citrate at pH 4.8. 5 μl of Na^+,K^+-ATPase suspension was diluted in 1 ml of assay medium containing 4 mM ATP, 100 mM NaCl, 25 mM KCl, 3.9 mM $MgCl_2$, 0.2 mM EDTA, 30 mM imidazole and 30 mM glycylglycine, pH 7.2, and was incubated for 5 min on ice. It was then incubated for 10 min at 37°C, and the ATP hydrolysis was stopped by adding 0.5 ml of 5% sodium dodecyl sulfate. Inorganic phosphate was assayed by the method of Hegyvary et al. [13].

3. RESULTS

Membrane-bound Na^+,K^+-ATPase suspended in a neutral medium was crystallized by simply dialysing against acidic buffers (Fig. 1). The best 2D crystals were obtained in 50 mM sodium citrate at pH 4.8 (Fig. 2A). The image of these negatively stained crystals showed reflections higher than 20 Å in optical diffraction, as shown in Fig. 2B. This image shows that the crystalline arrays were formed where two membrane sheets stack together, as schematically shown in Fig. 2C. In this figure, the shaded portion indicates the stacking region. The molecules appeared to condense in such regions and there were no crystalline arrays on single membranes. The image of the ice-embedded 2D crystal and its optical diffraction pattern are shown in Fig. 2D and E, respectively. The farthest Friedel pairs are circled in both patterns; 18.7 Å and 22.2 Å for stained and ice-embedded crystals, respectively.

The images of both the negatively stained and ice-embedded 2D crystals showed essentially the same lattice constants and symmetry; a = 111.1 Å, b = 65.4 Å, γ = 103.5°, and a = 66.2 Å, b = 108.7 Å, γ = 104.2°, respectively, and a p2 symmetry according to the computer analysis. This indicates that no serious deformation of the crystal during the staining procedure occurred. The processed image of the negatively stained 2D crystal, which is shown in Fig. 3A, indicates that there are two pear-shaped peaks in one unit cell. The processed image of the ice-embedded 2D crystal, which is essentially the same as that of the negatively stained image, is also shown in Fig. 3B. It showed a further detailed structure and were more promising. Especially, as indicated in Fig. 3B, there are two distinct small peaks in each main peak that is not readily recognizable in either Fig. 3A or the previously published filtered images of the same protein.

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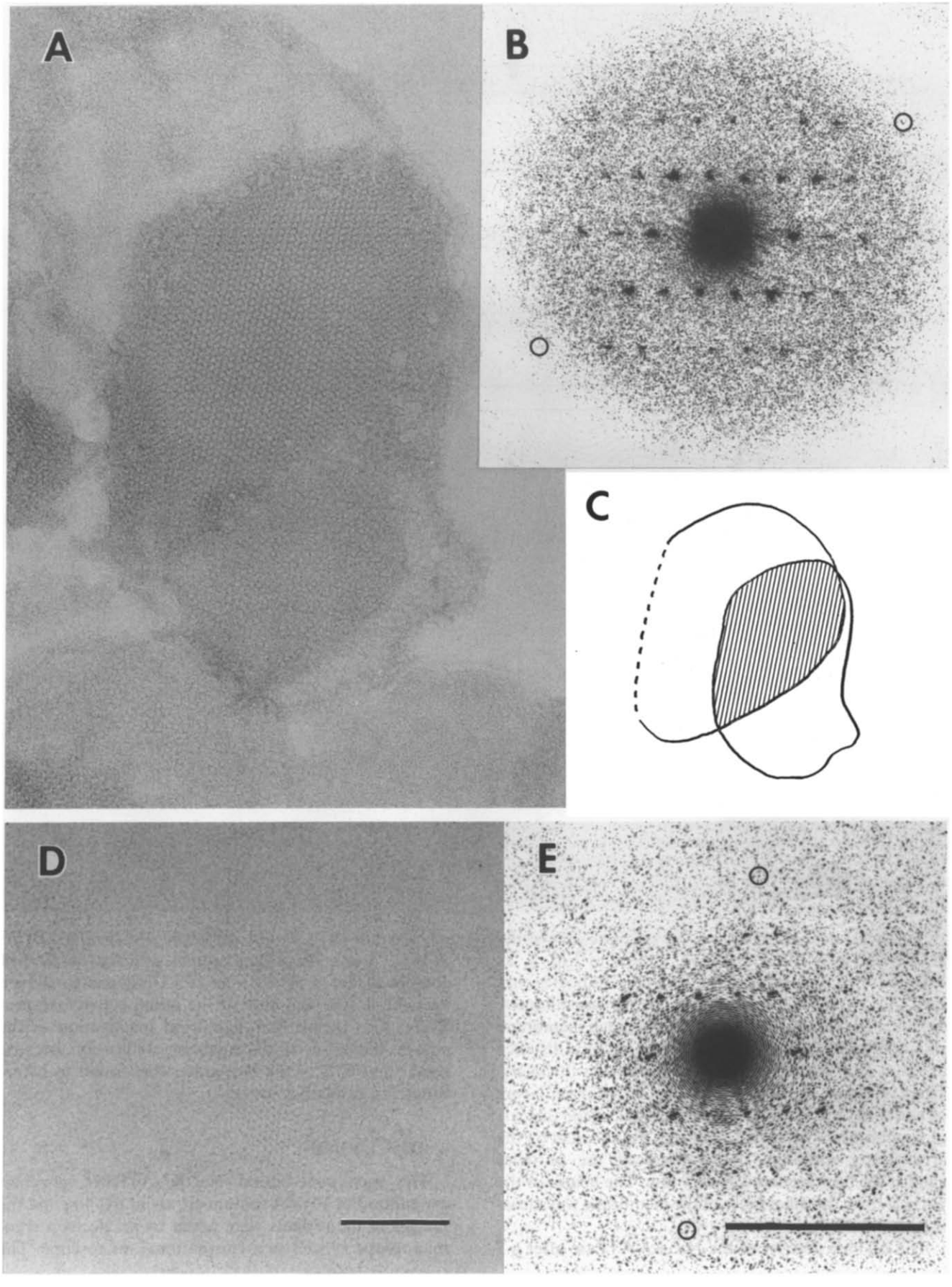
Fig. 2. (A) The image of negatively stained 2D crystal grown in 50 mM sodium citrate at pH 4.8, and (B) its optical diffraction. (C) A schematic drawing of the part of the 2D crystal in A. (D) The image of ice-embedded 2D crystal and (E) its optical diffraction. Bar = 0.1 μm in the images and 20 $Å^{-1}$ in the diffraction patterns.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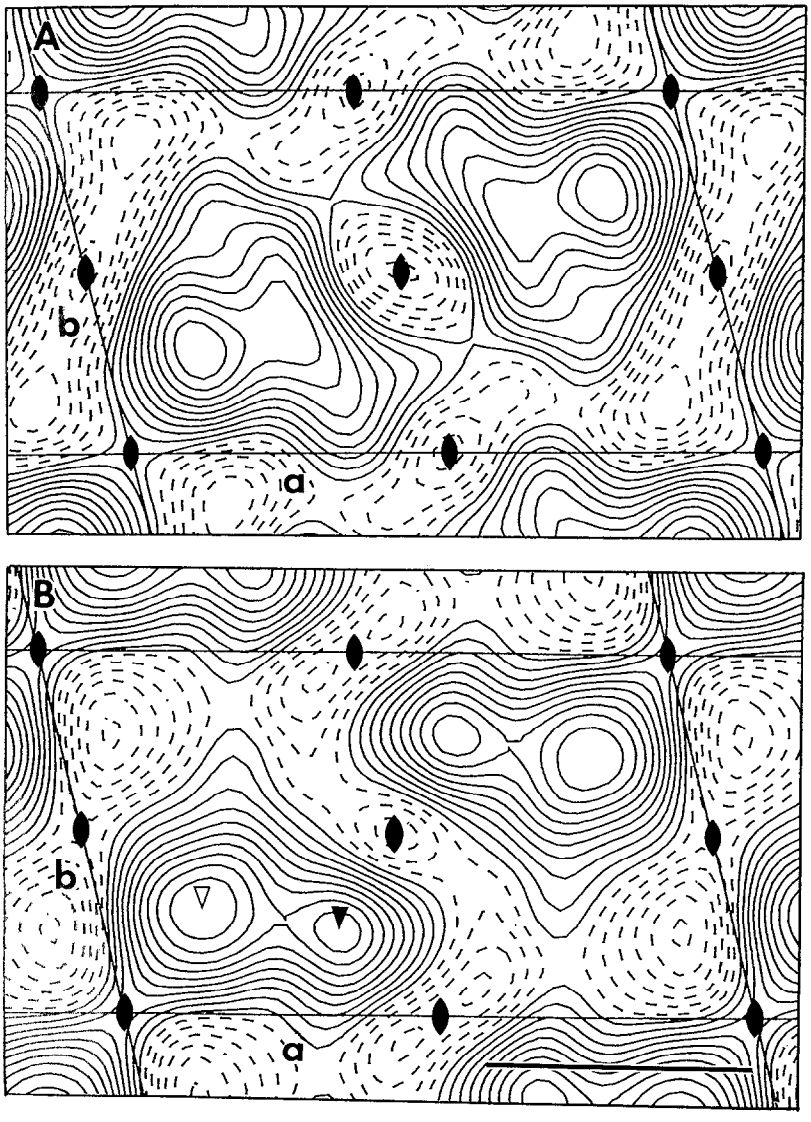

Fig. 3. Filtered images of (A) the negatively stained 2D crystal and (B) the ice-embedded crystal. The unit cell is drawn in both images; **a** and **b** are the 2 unit cell axes and the pointed ellipses are at the location of 2-fold rotation axes. Two small peaks are indicated as peak 1 and peak 2 by an open and solid arrowhead, respectively. Bar = 50 Å.

In the case of ice-embedding, the edge-on view of the 2D crystals was often observed, shown in Fig. 4, as reported by Sakata et al. [14]. This view indicates that each 2D crystal is composed of a pair of membranes, as indicated by the arrows in the figure. Each membrane showed double-layered contrast separated by a distance of about 35 Å that corresponds to the bilayer thickness. There is a space of 130 Å width between the inner layers of both bilayers. There are some particles inside the pair of membranes arranged in a zig-zag, as indicated by the arrowheads, although the contrast of the particles against the background was very low. The height of the particle from the inner layer of the membrane was measured as 40–90 Å.

The ATPase activity of the Na^+,K^+-ATPase after a 2 day incubation in 20 mM imidazole and 16 mM HEPES at pH 7.6 was 34.6 U/mg protein, and that in 50 mM sodium citrate at pH 4.8 was 28.9 U/mg protein, which was about 70% and 60% of the initial activity, respectively. This means that significant inactivation of the enzyme did not occur during the crystallization, because more than 60% of the molecules were found to be arranged in crystalline arrays.

4. DISCUSSION

The membrane-bound Na^+,K^+-ATPase was best crystallized in 50 mM sodium citrate at pH 4.8, and the images of the crystals were taken by an electron cryomicroscope as well as a conventional microscope. The

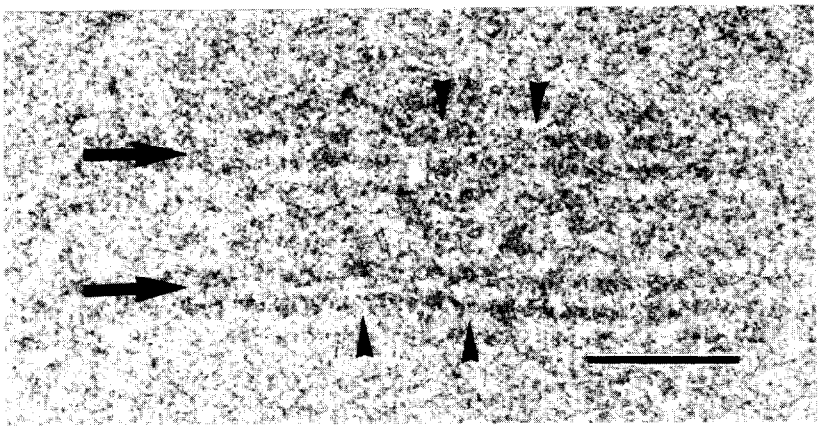

Fig. 4. Edge-on view of the 2D crystal. The arrows indicate the two membranes forming the crystal. The arrowheads indicate the particles arranged in a zig-zag in the space between the pair of membranes. Bar = 200 Å.

reconstituted Fourier map revealed the more detailed structure of the Na^+,K^+-ATPase when embedded in ice than when stained with uranyl acetate, although they are very similar to each other. In the single unit cell, there are two strong pear-shaped peaks that are related by a 2-fold rotation.

The crystalline arrays were localized within the area where the two membranes stack together with the distance of 130 Å. Even if the height of the protomer is the same as this distance, 130 Å, the projected area of the protomer onto the *ab*-plane can be calculated to be about 1,500 $Å^2$ from the molecular weight of 150 kDa. Thus, it is not reasonable to confine more than two $\alpha\beta$-protomers in one strong peak in the filtered image.

Phase origin search revealed that the crystal symmetry is p2. However, some low magnification images showed a p1 symmetry of the crystals, probably because of slight tilting of the crystals or some deformation due to crystal contact with the carbon support. Therefore, the crystal might have pseudo-symmetry and this should be determined by further structure analysis, including 3D structure reconstruction.

A possible packing of the protomers between the two sheets of the membranes can be expected by the fact that the 2D crystals are formed in the area where two membranes stack together (Fig. 2C) and the edge-on view (Fig. 4). The images of particles arrange in a zig-zag, and the distance between the center of the particle and the inner layer of the membrane is alternately 40 Å and 90 Å. The particles seen between the pair of membranes may be the extracellular portion of the protomer (58% of the whole molecular mass [1]), because there are no such large particles outside the membranes. Therefore, the protomer protruding upwards from the lower membrane faces upside-down to the protomer protruding downwards from the upper membrane. Thus, this interaction may play an important role in the 2D crystallization of Na^+,K^+-ATPase.

In the reconstituted map of the ice-embedded crystal the main peak due to the protomer can be divided into two small peaks, peak 1 and peak 2, in each protomer as indicated by arrowheads in the Fig. 3B, which is not as apparent in the reconstituted map of the negatively stained crystal, or those reported previously [4–7]. This is due to the difference in the imaging mechanism; in the case of negative stain, uranyl acetate cannot penetrate the lipid bilayer, which results in no density of the intramembranous part of the protein on the image, whereas in the case of ice-embedding, all the parts, including the intramembranous region, can be imaged. Therefore, the separation between the small peaks or the imaging of the intramembranous region of the protein can be achieved only by the imaging of the whole protomer using the electron cryo-microscope. For the first time the image of Na^+,K^+-ATPase has been taken clearly without staining.

Na^+,K^+-ATPase takes the two main different conformations, E_1 and E_2 [1]. When crystallized with vanadate ion, the enzyme takes the E_2 conformation, K^+-form [4]. In the acidic medium, it is thought to be in the E_1 conformation, Na^+-form, due to hydrogen ions binding to the enzyme in place of sodium ions [15]. There is also the opposite conclusion that Na^+,K^+-ATPase takes the E_2 at acidic pH while the E_1 conformation at alkaline pH [16,17]. Other studies, such as [18,19], showed that there was no apparent evidence to decide which conformation, E_1 or E_2, was realized at acidic pH. Therefore, we cannot conclude the conformation in the acidic medium and it can only be elucidated after the structure of Na^+,K^+-ATPase is solved at a higher resolution.

Although our reconstituted maps gave more detailed information than those presented previously, the size of the crystal is still too small to attain a high resolution structure analysis of Na^+,K^+-ATPase that will resolve secondary or higher order structure. However, this crystallization in acidic medium solved the three problems that prevented the high resolution structural analysis of

Na^+,K^+-ATPase. The molecules frequently appeared in highly ordered arrays, and the 2D crystals have a unique lattice constant and less crystalline defects. Moreover, this method does not destroy the enzymatic activity, which indicates that the crystallization does not result in the denaturation of the enzyme, and that the solved structure is that of the native molecule. Therefore, further structural analysis with current crystals and also the improvement of the crystal size will allow us further insight into the structure and function of Na^+,K^+-ATPase.

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